METHOD FOR MODIFICATION OF NMDA RECEPTORS THROUGH INHIBITION OF SRC

FIELD OF THE INVENTION

The instant invention relates generally to protein-protein interactions that regulate intra and intercellular communication; particularly to methods for modification of protein-protein interactions; and most particularly to a method for modifying the activity of NMDA (N-methyl-D-aspartate) receptors located in cells by inhibition of the interaction of the unique domain of the tyrosine kinase Src enzyme with proteins of the NMDAR complex.

BACKGROUND OF THE INVENTION

Excitatory transmission at central synapses is primarily mediated by the amino acid glutamate acting through postsynaptic ionotropic receptors (Dingledine et al. Pharmacological Review 51:7-61 1999). The N-methyl-D-aspartate receptor (NMDAR) is one such type of ionotropic glutamate receptor (Dingledine et al. Pharmacological Review 51:7-61 1999). NMDARs are multiprotein complexes located at excitatory synapses within the postsynaptic density (PSD) comprised of the core channel subunits together with associated scaffolding and regulatory proteins that control

receptor localization, ionic flux through the receptor and downstream signaling events (Scannevin et al. Nature Reviews Neuroscience 1:133-141 2000; Sheng et al. Annual Review of Physiology 62:755-778 2000). NMDAR's are crucial for central nervous system (CNS) development, neuroplasticity and pathophysiology (Dingledine et al. Pharmacological Review 51:7-61 1999; Sheng et al. Science 298:776-780 2002). Multiple factors regulate NMDAR function, including dynamic cycling of protein phosphorylation and dephosphorylation at serine/theronine or tyrosine residues (Wang et al. Nature 369:233-235 1994; Smart Current Opinion in Neurobiology 7:358-367 1997). The Src protein is one such factor that modulates the activity of the NMDARs (Yu et al. Science 275:674-678 1997; Lu et al. Science 279:1363-1368 1998; Yu et al. Nature 396:469-474 1998).

The non-receptor protein tyrosine kinase Src is a ubiquitous enzyme with key roles in diverse development, physiological and pathological processes (Brown et al. Biochim. Biophys. Acta 1287:121-149 1996). Domains identified in Src-the Src homology 3 (SH3) domain, the SH2 domain and the SH1 (catalytic) domain are signature regions that have been used to define highly-conserved protein modules found in a wide variety of signaling proteins (Pawson Nature 373:573-580 1995). In addition to these highly-conserved regions, Src

also contains a region of low sequence conservation and unknown function, termed the unique domain.

Src is highly expressed in the CNS, functioning to regulate glutamatergic neurotransmission and synaptic plasticity (Ali et al. Current Opinion in Neurobiology 11:336-342 2001). At glutamatergic synapses, Src modulates the activity of NMDARs (Yu et al. Science 275:674-678 1997; Lu et al. Science 279:1363-1368 1998; Yu et al. Nature 396:469-474 1998). Src represents a point through which multiple signaling cascades from G-protein coupled receptors (Luttrell et al. Journal of Cell Science 115:455-465 2002), Eph receptors (Henderson et al. Neuron 32:1041-1056 2001; Takasu et al. Science 295:491-495 2002; Murai et al. Neuron 33:159-162 2002) and integrins (Lin et al. Journal of Neurophysiology 89:2874-2878 2003; Kramar et al. Journal of Biological Chemistry 278:10722-10730 2003) converge to upregulate NMDAR channel activity, thus mediating essential neuronal excitation. The upregulation of NMDAR activity by Src is necessary for long-term potentiation (LTP) of synaptic transmission at Schaffer collateral-CA1 neuron synapses in the hippocampus(Ali et al. Current Opinion in Neurobiology 11:336-342 2001), the predominant cellular model for learning and memory (Kandel Science 294:1030-1038 2001).

However, abnormal regulation of NMDARs can have numerous

pathologic effects; most resulting from the production of nitric oxide, a signaling molecule which mediates excitotoxicity (Dawson et al. Proceedings of the National Academy of Science USA 88:6368 1991). NMDARS mediate ischemic brain injury, as seen, for example in stroke and traumatic injury (Simon et al. Science 226:850 1984). In addition, abnormal NMDAR regulation has been implicated in Alzheimer's disease, Parkinson's disease (Coyle et al. Science 262:689 1993), schizophrenia (Hirsch et al. Pharmacology Biochemistry and Behavior 56(4):797-802 1997), epilepsy (US Patent 5,914,403), glaucoma (US Application 2002 0077322 A1) and chronic pain (Guo et al. Journal of Neuroscience 22:6208-6217 2002).

Although NMDARs are implicated in numerous pathological conditions, non-selective blocking of their function is deleterious, since complete blockade of synaptic transmission mediated by NMDA receptors is known to hinder neuronal survival (Ikonomidou et al. Lancet: Neurology 1:383-386 2002; Fix et al. Experimental Neurology 123:204 1993; Davis et al. Stroke 31:347 2000; Morris et al. Journal of Neurosurgery 91:737 1999).

Additionally, inhibition of Src kinases may also have deleterious results. Since kinases play a part in the regulation of cellular proliferation, they are frequently

targeted for the development of new cancer therapies. The majority of these therapies inhibit function of the kinase catalytic domain, which is often highly conserved between distinct kinases. Thus, inhibition of Src in the CNS with a standard kinase inhibitor may cross-react with additional kinases and adversely affect normal neuronal functions.

Considering the above-mentioned deleterious effects resulting from direct blockage of NMDARs and/or indirect inhibition of NMDARs through the use of kinase inhibitors, it is clear that there remains a need in the art for a method of modifying NMDARs which can attenuate downstream NMDAR signaling, without completely blocking, ion-channel activity.

DESCRIPTION OF THE PRIOR ART

Since the NMDA receptor is critical to both normal neuronal function and pathology, there are many known methods for modification of NMDA receptors; several examples of which are noted below.

US Patent 5,888,996 (David Farb) discloses a method for inhibiting NMDA glutamate receptor-mediated ion channel activity by treatment with an effective amount of a derivative of pregnenolone sulfate. This patent also discloses a method for modulating/altering excitatory

glutamate-mediated synaptic activity by contacting neurons with pregnenolone sulfate or a derivative of pregnenolone sulfate.

US Patent 5,914,403 (Nichols et al.) discloses agents capable of modifying neuroexcitation through excitatory amino acid antagonists; in particular quinolinic acid derivatives antagonistic to a glycine binding site in the NMDAR complex. The agents disclosed by Nichols et al. have anticonvulsant activity.

US Patent 4,994,446 (Sokolovsky et al.) discloses a drug system comprising a MK-801/PCP type drug administered in combination with/or in sequence with excitatory amino acids such as, glutamate, glycine, aspartate and analogs thereof. The excitatory amino acids facilitate binding of the drug to the NMDAR channels. This drug system has anticonvulsant activity and can alleviate brain damage due to stroke.

US Patent 6,653,354 (Franks et al.) discloses a method for reducing the level of NMDAR activation by use of the NMDA antagonist, xenon to inhibit synaptic plasticity. The xenon composition of Franks et al. also acts as a neuroprotectant.

US Patent Application 2002 0123510 A1 (Chenard et al.) discloses a method for treatment of traumatic brain injury (TBI) and stroke by administration of a NR2B subtype selective NMDAR antagonist in combination with either of the

following agents; sodium channel antagonist, nitric oxide synthase inhibitor, glycine site antagonist, potassium channel opener, AMPA/kainate receptor antagonist, calcium channel antagonist, GABA-A receptor modulator, anti-inflammatory agent or a thrombolytic agent. These agents either protect neurons from toxic insult, inhibit inflammatory responses after brain damage or promote cerebral reperfusion after hypoxia or ischemia.

Planells-Cases et al. (Mini Review of Medicinal Chemistry 3(7):749-756 2003) disclose that small molecule antagonists of the NMDAR are useful for the treatment of neuropathic pain caused by injury to the peripheral or central nervous system.

US Patent Application 2002 0077322 A1 (George Ayoub) discloses methods for protecting neuronal cells from glutamate-induced toxicity, such as that which occurs in ischemia and glaucoma, by increasing the activity of a cannabinoid agonist which binds specifically to a cannabinoid receptor.

US Patent Application 2003 0050243 A1 (Michael Tymianski) discloses a method for inhibition of binding between NMDARs and neuronal proteins. The inhibition is created by administration of a peptide replacement of either an NMDAR or neuronal protein interaction domain. Post-

synaptic density protein 95 (PSD-95) couples NMDARs to pathways mediating excitotoxicity and ischemic brain damage. The method of Tymianski involves transducing neurons with peptides that bind modular domains on either side of the NMDAR/PSD-95 interaction complex. This transduction attenuates downstream NMDAR signaling without blocking receptor activity, protects cortical neurons from ischemic insult and reduces cerebral infarction in rats exposed to transient focal cerebral ischemia. This treatment was effective in the rats when applied before or one hour after the ischemic insult. (Aarts et al. Science 298:846-850 2002) also discloses the research described in US Patent Application 2003 0050243 A1.

As is exemplified by the examples listed above, the majority of known methods for modification of NMDA receptors generally involve administration of receptor antagonists which inhibit receptor function completely. The instant inventors are the first to modify the NMDAR by inhibiting the interaction of the unique domain of the tyrosine kinase Src enzyme with NADH dehydrogenase subunit 2 (ND2); thus preventing Src upregulation of the NMDAR by preventing binding between Src and ND2.

SUMMARY OF THE INVENTION

Src-mediated upregulation of NMDAR activity is prevented by peptide fragments of the Src unique domain and by a unique domain-binding antibody (Yu et al. Science 275:674-678 1997; Lu et al. Science 279:1363-1368 1998) leading to the hypothesis that the upregulation of NMDAR function by Src depends on an interaction between a region in the unique domain of Src and an unknown protein in the NMDAR complex (Ali et al. Current Opinion in Neurobiology 11:336-342 2001). In order to test the hypothesis, the instant inventors searched for proteins that may interact with the unique domain of Src and may thereby mediate the interaction between this kinase and NMDARs. These proteins were generally termed "SUDAPIs" (Src unique domain anchoring protein inhibitors)by the instant inventors since they anticipate that other such inhibitors may exist which exhibit identical functions.

As a result of their search, the instant inventors became the first to identify NADH dehydrogenase subunit 2 (ND2;nucleotide sequence SEQ ID NO:8 and amino acid sequence SEQ ID NO:9) as a Src unique domain-interacting protein. ND2 functions as an adapter protein anchoring Src to the NMDAR complex, thus permitting Src-mediated upregulation of NMDAR activity. The instant inventors identified a region of the Src unique domain which interacts with ND2; a region located approximately at amino acid positions 40-49 of the Src

protein(SEQ ID NO:1). The exogeneous peptide inhibits the ability of ND2 to anchor the Src protein to the NMDAR complex. This peptide, approximately 10 amino acids in length, has been named "SUDAPI-1" by the instant inventors, since it is the first such peptide discovered which functions to inhibit the Src unique domain anchoring protein. Administration of this exogeneous peptide prevents ND2 interaction with the Src unique domain; thus inhibiting Src-mediated upregulation of NMDAR activity. Since this peptide alone cannot cross the cell membrane to enter the cellular interior, it is combined with a carrier capable of penetrating the cell membrane. Illustrative, albeit non-limiting examples of carriers are peptides derived from viral transduction domains, such as the TAT domain derived from the Human Immunodeficiency Virus (HIV) and VP22 derived from the Herpes Simplex Virus, arginine-rich peptides, fusogenic antennapedia peptides derived from Drosophilia and lipids. Lipids can facilitate crossing of the cell membrane by enclosing the peptide in a lipid vesicle or liposome (lipid transfection protocol) or the peptide can be directly modified with lipid groups. Use of the HIV-Tat domain peptide as a carrier is exemplified in the Examples described herein. SUDAPI-1 fused to the HIV-Tat domain is designated "TSUDAPI-1" (SEQ ID NO:2). The NMDAR activity is evoked by glutamate

and is additionally regulated by many distinct pathways other than the Src pathway. Inhibition of Src suppresses but does not completely inhibit the NMDAR as is apparent from the electrophysiologic measurements of receptor activity shown in Figures 5D-F. Thus, the instant invention provides methods and compositions for modifying NMDAR function without completely blocking the receptor or adversely affecting other neuronal proteins with the use of generalized kinase inhibitors. These methods and compositions may be used to ameliorate diseases and/or other conditions related to NMDAR signaling. Illustrative, albeit non-limiting examples of such diseases and/or other conditions are stroke, hypoxia, ischemia, multiple sclerosis, Huntington's chorea, Parkinson's disease, Alzheimer's disease, hyperglycemia, diabetes, traumatic injury, epilepsy, grand mal seizures, spasticity, cerebral palsy, asthma, cardiac arrest, macular degeneration, mental diseases, schizophrenia, AIDS dementia complex, other dementias, AIDS wasting syndrome, inflammation, pain, opioid addiction, cocaine addiction, alcohol addiction, other conditions associated with substance abuse and anorexia. An example of such amelioration is illustrated in Example 7 wherein pain behaviors are reduced in rats treated with the composition of the instant invention prior to undergoing the formalin test.

Src upregulation of the NMDAR is involved in the pathway of long-term potentiation (LTP)(Huang et al. Neuron 29:485-496 2001; Lu et al. Science 279:1363-1367 1998). Since LTP is considered a model for learning and memory, the compositions of the instant invention are contemplated for use in methods which elucidate mechanisms of learning and memory and/or enhance learning and memory.

The NMDAR is expressed almost exclusively in neurons; however the interaction between Src and ND2 was shown to occur in multiple and diverse tissues (Example 8 and Figures 10A-B). Thus, the instant inventors hypothesize that the Src-ND2 interaction has functions other than regulation of NMDAR's and contemplate that the compositions of the instant invention can be used in methods for the general inhibition of Src in multiple cell types.

Accordingly, it is an objective of the instant invention to provide a method for modifying NMDAR interaction with non-receptor tyrosine kinase Src in any cell which expresses the NMDAR by providing a composition including at least one SUDAPI and administering the composition to the cell in an amount effective to achieve modification of the NMDAR interaction with non-receptor tyrosine kinase Src in the cell wherein said modification ameliorates a disease or condition related to NMDAR signaling. The methods and compositions of

the instant invention are particularly suited to use with cells of the nervous system but can also be used with any cell which expresses the NMDAR.

It is another objective of the instant invention to provide a pharmaceutical composition for modifying NMDAR interaction with non-receptor tyrosine kinase Src in cells comprising at least one SUDAPI combined with a pharmacologically acceptable solution or carrier.

It is also an objective of the instant invention to provide a method for modifying NMDAR interaction with non-receptor tyrosine kinase Src in any cell which expresses the NMDAR by providing a composition including SUDAPI-1 and administering the composition to the cell in an amount effective to achieve modification of the NMDAR interaction with non-receptor tyrosine kinase Src in the cell wherein said modification ameliorates a disease or condition related to NMDAR signaling.

It is another objective of the instant invention to provide a pharmaceutical composition for modifying NMDAR interaction with non-receptor tyrosine kinase Src in cells comprising SUDAPI-1 combined with a pharmacologically acceptable solution or carrier.

It is yet another objective of the instant invention to provide a method for modifying NMDAR interaction with non-

receptor tyrosine kinase Src in any cell which expresses the NMDAR by providing a composition including TSUDAPI-1 and administering the composition to the cell in an amount effective to achieve modification of the NMDAR interaction with non-receptor tyrosine kinase Src in the cell wherein said modification ameliorates a disease or condition related to NMDAR signaling.

It is still another objective of the instant invention to provide a pharmaceutical composition for modifying NMDAR interaction with non-receptor tyrosine kinase Src in cells comprising TSUDAPI-1 combined with a pharmacologically acceptable solution.

It is another objective of the instant invention to provide an isolated peptide (ND2.1; SEQ ID NO:7) which interacts with the Src unique domain to anchor Src to the NMDAR complex thus permitting Src-mediated upregulation of NMDAR activity.

It is still another objective of the instant invention to provide a method for inhibiting non-receptor tyrosine kinase Src in cells expressing non-receptor tyrosine kinase Src by providing a composition including at least one SUDAPI and administering the composition to the cells in an amount effective to achieve inhibition of non-receptor tyrosine kinase Src in the cells.

It is another objective of the instant invention to provide a pharmaceutical composition for inhibiting non-receptor tyrosine kinase Src in cells comprising at least one SUDAPI combined with a pharmacologically acceptable solution or carrier.

It is another objective of the instant invention to provide a composition useful in methods for elucidating the mechanisms of learning and memory.

It is yet another objective of the instant invention to provide a composition useful in methods for enhancing learning and memory.

Other objectives and advantages of the instant invention will become apparent from the following description taken in conjunction with the accompanying drawings wherein are set forth, by way of illustration and example, certain embodiments of the instant invention. The drawings constitute a part of this specification and include exemplary embodiments of the present invention and illustrate various objects and features thereof.

ABBREVIATIONS AND DEFINITIONS

The following list defines terms, phrases and abbreviations used throughout the instant specification. Although the terms, phrases and abbreviations are listed in

the singular tense the definitions are intended to encompass all grammatical forms.

As used herein, the term "modification" refers to any action and/or treatment which alters the function of a protein.

As used herein, the term "inhibition" refers to any action and/or treatment which operates against the full activity of a protein thus reducing and/or completely suppressing protein function.

As used herein, the term "interaction" refers to an action wherein two substances in close physical proximity act upon each other.

As used herein, the term "anchor" means to stabilize or secure firmly in place.

As used herein, the term "isolated peptide" refers to a peptide which has been "altered by the hand of man" and separated from the co-existing materials of its natural state. An isolated peptide has been changed or removed from its original environment or both.

As used herein, the abbreviation "CNS" refers to the central nervous system, which includes the brain, cranial nerves and the spinal cord.

As used herein, the abbreviation "PNS" refers to the peripheral nervous system, which is the network of nerves

that connect the CNS to organs, muscles, vessels and glands.

As used herein, the term "excitatory neurotransmission" refers the passage of signals from one neuron to another via chemical substances or electrical impulses.

As used herein, the abbreviation "NMDAR" refers to the N-methyl-D-aspartate receptor, an ionotropic cation-ion specific, ligand-gated(glutamate-gated) ion channel which is activated by NMDA or NMDA-like ligands(agonist activation)such as glutamate. The NMDAR is a multi-protein complex including the core channel subunits with associated scaffolding and regulatory proteins, located in the excitatory synapses in the post-synaptic density. Activation of the receptor opens the channel to allow cations (Ca^{+2}, Na^{+} and K^{+})to cross the cellular membrane. "Upregulation of NMDAR activity" refers to the enhanced opening of the receptor ion channels.

As used herein, the abbreviation "PSD" refers to the post-synaptic density, a specialized portion of the neuronal cytoskeleton, located near the post-synaptic membrane. The PSD provides a support matrix for signal transduction.

As used herein, the term "Src" refers to a protein exhibiting tyrosine-specific kinase activity. The Src protein is involved in controlling diverse cellular functions, including regulation of NMDAR activity.

As used herein, the abbreviation "LTP" refers to long term potentiation, an activity-dependent persistent enhancement of synaptic transmission which is considered a model of learning and memory. The biochemical signaling cascade which results in LTP involves the activation of Src which in turn, activates NMDARs.

As used herein, the abbreviation "ND2" refers to NADH dehydrogenase subunit 2, a subunit of mitochondrial Complex I. The instant inventor was the first to recognize that ND2 is present in the PSD and acts as an adaptor protein for anchoring Src to the NMDAR complex.

As used herein, the abbreviation "SUDAPI" refers to any substance which functions as a Src unique domain anchoring protein inhibitor.

As used herein, the abbreviation "SUDAPI-1" refers to the first Src unique domain anchoring protein inhibitor discovered by the instant inventors. SUDAPI-1 is a peptide, generally 10 amino acid residues in length corresponding approximately to amino acid positions 40-49 of the Src unique domain (SEQ ID NO:1).

As used herein, the phrase "corresponding approximately to amino acid positions 40-49 of the Src unique domain" refers to the slight difference which is possible in amino acid position numbering of the Src protein due to species

variations and conventions within the art regarding whether the first methionine counts as a residue or not.

As used herein, the abbreviation "TSUDAPI-1" refers to SUDAPI-1 which is combined with the carrier peptide, HIV-Tat (SEQ ID NO:2).

As used herein, the term "carrier" refers to any substance which is attached to another substance which alone cannot traverse the cell membrane to enter the cellular interior. The carrier substance functions to carry this other substance through the cellular membrane into the cellular interior. Illustrative, albeit non-limiting examples include lipids and peptides having transducing and/or fusogenic ability.

As used herein, the term "HIV-Tat" refers to the transduction domain of the human immunodeficiency virus (HIV); the causative agent of Acquired Immunodeficieny Syndrome (AIDS). HIV-Tat peptide is often used as a carrier to transport molecules into cells.

As used herein, the term "VP22" refers to a transduction domain of the herpes simplex virus. VP22 peptide is often used as a carrier to transport molecules into cells.

As used herein, the term "antennapedia" refers to peptides derived from Drosophilia which have fusogenic ability. Antennapedia peptide is often used as a carrier to

transport molecules into cells.

The phrase "pharmaceutically acceptable" is used herein as described in US 6,703,489. "Pharmaceutically acceptable" means approved by a regulatory agency or listed in a generally approved pharmacopeia for use in animals and humans. Solutions are usually preferred when a composition is administered intravenously. Illustrative, albeit non-limiting examples of pharmaceutically acceptable solutions include water, oils, saline, aqueous dextrose and glycerol.

As used herein, the phrase "amount effective" refers to an amount of a composition sufficient to elicit a change in activity of the NMDAR.

As used herein, the phrase "ameliorate a disease and/or condition" refers to an action which causes symptoms of a disease and/or condition to improve or become better.

As used herein, the abbreviation "SH" refers to a Src homology domain; regions that have been used to define highly-conserved protein modules found in a wide variety of signaling proteins (T. Pawson Nature 373:573-580 1995).

As used herein, the phrase "unique domain" refers to a Src domain having low sequence conservation and unknown function.

As used herein, the abbreviation "ND4" refers to NADH degydrogenase subunit 4, an oxidoreductase protein, a

component of mitochondrial Complex I(JE Walker Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov et al. Biochemistry 39:7229-7235 2000; Sazanov et al. Journal of Molecular Biology 302:455-464 200).

As used herein, the abbreviation "Cyto 1" refers to cytochrome *c* oxidase subunit 1, an inner mitochondrial membrane protein that is part of Complex IV (Marusich et al. Biochim. Biophys. Acta 1362:145-159 1997).

As used herein, the abbreviation "mEPSCs" refers to miniature excitatory post-synaptic currents, a type of excitatory neurotransmission.

The terms "SUDAPI-1" and "Src40-49" are used interchangeably herein (SEQ ID NO:1).

The terms "TSUDAPI-1"; "Src40-49-Tat"; "Src40-49-HIV-Tat"; "Tat-Src40-49" and "HIV-Tat-Src40-49" are used interchangeably herein (SEQ ID NO:2).

The terms "Src40-58" and "scrambled Src40-58" are used repeatedly throughout and refer to peptides comprising amino acid residues 40-58 of SEQ ID NO:4.

The term "Src49-58" is used repeatedly throughout and refers to a peptide comprising amino acid residues 49-58 of SEQ ID NO:4.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-E show the results of experiments evidencing that ND2 is a Src unique domain-interacting protein.

Figures 2A-E show the results of experiments evidencing that ND2 is present at the post-synaptic density.

Figures 3A-B show the results of experiments evidencing that ND2 interacts with Src at the post-synaptic density.

Figures 4A-G show the results of experiments evidencing that ND2 interacts with Src at the NMDAR complex.

Figures 5A-F show the results of experiments evidencing that blocking expression of ND2 prevents Src-dependent regulation of NMDA receptor activity. Figure 5F shows amino acid residues 4-7 of SEQ ID NO:5 (pY)EEI.

Figures 6A-C show the results of experiments evidencing that the Src40-49 (SUDAPI-1) peptide specifically interacts with the ND2.1 peptide.

Figures 7A-D show results of experiments showing the effects of TSUDAPI-1 on 2.5% formalin induced flinching or biting/licking behaviors.

Figures 8A-D show results of experiments showing the effects of HIV-TAT on 2.5% formalin induced flinching or biting/licking behaviors.

Figures 9A-B show SEQ ID NOS:6 and 7; Figure 9A shows the nucleotide sequence encoding recombinant ND2.1

protein(SEQ ID NO:6); Figure 9B shows the amino acid sequence of recombinant ND2.1 protein (SEQ ID NO:7).

Figures 10A-B show immunoblots evidencing that ND2 and Src interact in multiple, diverse tissues.

DETAILED DESCRIPTION OF THE INVENTION

EXAMPLE 1

NADH dehydrogenase subunit 2 (ND2) is a Src unique domain-binding protein.

A yeast two-hybrid screen of a fetal brain library using bait constructs containing the murine Src unique domain was conducted in order to search for proteins that interact with the Src unique domain.

cDNAs encoding amino acids 4-82 (the Src unique domain) and amino acids 4-150 (the Src unique and SH3 domains) of murine n-Src were ligated into pEG202 (Gyuris et al. Cell 75:791-803 1993) to create two expression vectors encoding in frame LexA fusions containing the Src unique domain (the nucleotide sequence encoding Src is SEQ ID NO:3 and the amino acid sequence is SEQ ID NO:4). The bait constructs were then sequenced. Both baits were tested to ensure that the baits did not activate transcription of the reporters in the

absence of prey and that both could enter the nucleus and bind to LexA operators. To create the selection strains for screening, each bait plasmid was individually transformed into the yeast strain EGY48. EGY48 has an integrated Leu2 selectable marker regulated by 6 LexA operator repeats, and carries a reporter plasmid with the lacZ gene regulated by 8 LexA operator repeats. Bait-prey interactions that occur with low affinity result in activation of the Leu2 reporter gene only, whereas high affinity interactions result in activation of both the Leu2 and lacZ reporter genes, allowing for double selection of prey. The selection strain was transformed with a representative activation-tagged cDNA prey fusion library constructed using ~1 kilobase EcoRI fragmented poly A(+) RNA from human fetal brain. Yeast transformed with the prey library (approximately 1.1 x 10^6 clones) were screened by double selection on X-gal Leu^- medium. Prey cDNAs encoding proteins that interacted with the bait were isolated and sequenced.

Src, Fyn, and ND2 recombinant proteins were prepared. The cDNAs encoding the SH3 and SH2 domains of mouse n-Src and Fyn were PCR subcloned, ligated in frame into pGEX4T-1 (Amersham Pharmacia Biotech, Baie d'Urfé, Québec), and sequenced. These plasmids, as well as plasmids encoding the unique domains of Src and Fyn in pGEX2T'6, were transformed

into BL21 bacteria, and GST fusion proteins were purified by glutathione affinity chromatography. To create the ND2.1, ND2.2, and ND2.3 GST fusion proteins, cDNAs encoding amino acids 239-321 (ND2.1-GST; SEQ ID NO:7), amino acids 189-238 (ND2.2-GST; SEQ ID NO:11), and amino acids 1-188 (ND2.3-GST; SEQ ID NO:13) of human ND2 were PCR subcloned and ligated into pGEX4T-1 (the nucleotide sequence encoding ND2 is SEQ ID NO:8 and the amino acid sequence is SEQ ID NO:9; the nucleotide sequences encoding ND2.1; ND2.2 and ND2.3 are SEQ ID NOS:6, 10 and 12,respectively). Using PCR-based single nucleotide mutagenesis, all cDNAs encoding ND2 fusion proteins were corrected for differences between mitochondrial and nuclear codons to prevent premature translation termination and protein truncation. All constructs were then confirmed by sequencing. The plasmids were transformed into bacteria, and GST fusion proteins were purified by glutathione affinity chromatography.

Detailed protocols for in vitro binding assays, pull down assays, immunoblotting, and co-immunoprecipitation techniques can be found in Pelkey et al. (Neuron 34:127-138 2002).

In two independent screens, cDNA fragments encoding overlapping regions within NADH dehydrogenase subunit 2 (ND2) were isolated (Figure 1A). ND2 is a 347 amino acid protein

(SEQ ID NO:9) that is a subunit of the inner mitochondrial membrane enzyme, NADH dehydrogenase (Complex I). ND2 is one of a group of seven oxidoreductase proteins that are encoded in the mitochondrial genome and which co-assemble with 35 nuclear encoded subunits to form Complex I. ND2 on its own lacks enzymatic activity (J.E. Walker Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov et al. Journal of Molecular Biology 302:455-464 2000; Sazanov et al. Biochemistry 39:7229-7235 2000). Figure 1A is a schematic diagram illustrating the domain structure of ND2, clones isolated from the yeast two hybrid screen, and recombinant GST-tagged fusion proteins. The lines point out the beginning of the oxidoreductase domain at amino acid position 23 and the end at amino acid position 197. Each clone and GST-fusion protein represent overlapping regions within ND2.

As yeast two-hybrid screening may reveal false positive protein-protein interactions, the interaction between Src and ND2 was observed using an independent methodological approach. Direct binding in vitro between ND2 and Src was tested using recombinant proteins. A series of GST fusion proteins comprised of portions of ND2 that spanned the overlapping region found with the yeast two-hybrid screen were made (Figure 1A). Importantly, the cDNAs encoding each of the ND2 fusion proteins were corrected for differences

between mitochondrial and nuclear codons so that the sequence of the ND2 portion of the fusion proteins was that which would have been produced by translation in the mitochondria. For example, Figure 9A shows the nucleotide sequence encoding recombinant ND2.1 protein (SEQ ID NO:6). Codons that are highlighted with bold type were altered by PCR-based single nucleotide mutagenesis. TGA was changed to TGG to prevent premature translation termination and protein truncation. GAA was changed to GAG to remove a restriction enzyme site. Numbers in parenthesis correspond to equivalent positions in the endogenous human ND2 nucleotide sequence. Figure 9B shows the amino acid sequence of recombinant ND2.1 protein(SEQ ID NO:7). Numbers in parenthesis correspond to equivalent positions in the endogenous human ND2 amino acid sequence. Each of the series of GST-fusion proteins was tested individually for interaction with the Src unique domain ("pull-down" assay). Figure 1B shows a blot of ND2-GST fusion proteins probed with biotinylated Src unique domain followed by a streptavidin-HRP conjugate. A GST fusion protein containing amino acids 239-321 of ND2 (ND2.1-GST; SEQ ID NO:7) was found that bound to the unique domain of Src (Figure 1B). In contrast, GST fusion proteins containing amino acids 189-238 (ND2.2-GST) or 1-188 (ND2.3-GST) of ND2 (ND2 protein sequence is SEQ ID NO:9) did not

bind to the Src unique domain. These results, together with those from the yeast two-hybrid screen, indicate that ND2 is a Src unique domain-binding protein. The results indicate further that the Src-binding portion of ND2 is contained within the region of amino acids 239-321(SEQ ID NO:7). This region of ND2 shows little conservation amongst the mitochondrially encoded oxidoreductase proteins and is outside the so-called "oxidoreductase domain", a signature region identified in all mitochondrially encoded subunits of NADH dehydrogenase (J.E. Walker Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov et al. Journal of Molecular Biology 302:455-464 2000; Sazanov et al. Biochemistry 39:7229-7235 2000) and some antiporters (Fearnley et al. Biochim. Biophys. Acta 1140:105-143 1992).

Another "pull-down" assay was conducted to determine whether the binding of ND2 might generalize to other domains of Src or to other Src family tyrosine kinases.

However, it was found that ND2.1-GST did not bind to either of the prototypic protein-protein interaction domains of Src, the SH2 or SH3 domains (Figure 1C). Figure 1C shows a blot of ND2.1-GST probed with biotinylated domains of Src and Fyn followed by streptavidin-HRP conjugate.

To examine the potential interaction of ND2 with other kinases of the Src family recombinant domains of Fyn were

tested, the protein most closely related to Src but which has little primary sequence conservation in the unique domain (Brown et al. Biochim. Biophys. Acta 1287:121-149 1996; T. Pawson Nature 373:573-580 1995). It was found that ND2.1-GST did not interact in vitro with the Fyn unique domain; nor did ND2.1 bind to the SH2 or SH3 domains of Fyn. Thus, the ND2.1 region does not interact with the SH2 or SH3 domains of Src or Fyn nor does it generally bind to the unique domain of Src family tyrosine kinases.

To investigate the possibility that Src and ND2 may interact in vivo, brain lysates were immunoprecipitated with antibodies directed against ND2 (anti-ND2) or against Src (anti-Src). It was found that immunoprecipitating Src led to co-immunoprecipitation of ND2 (Figure 1D). Figure 1D shows immunoblots of co-immunoprecipitates from brain homogenate probed with anti-ND2, anti-Src or anti-Fyn as indicated. Non-specific IgG was used as a negative control for immunoprecipitation. Fyn was readily detected in the brain homogenate used as a starting material for the co-immunoprecipitation (data not illustrated). Conversely, immunoprecipitating with anti-ND2 resulted in co-immunoprecipitation of Src. In contrast, anti-ND2 did not co-immunoprecipitate Fyn and neither ND2 nor Src was immunoprecipitated with a non-specific IgG (Figure 1D). As

an independent immunoprecipitation control it was found that ND2 was co-immunoprecipitated by anti-Src from $Src^{+/+}$ fibroblasts but not from $Src^{-/-}$ fibroblasts (Figure 1E). Figure 1E shows an immunoblot of co-immunoprecipitates from cultured $Src^{+/+}$ and $Src^{-/-}$ fibroblasts probed with anti-ND2. Non-specific IgG was used as a negative control for immunprecipitation, and immunoblotting of ND2 protein from both cell lines was used as a positive control. Thus, in addition to finding the ND2-Src unique domain interaction in two yeast two-hybrid screens and in vitro binding assays with recombinant proteins, it was found that ND2 and Src co-immunoprecipitated with each other, which together led to the conclusion that the ND2 is a Src unique-domain binding protein that may interact with Src in vivo.

EXAMPLE 2

ND2 is present in post-synaptic densities in brain.

Post-synaptic density proteins (Kennedy et al. Proceedings of the National Academy of Science USA 80:7357-7361 1983) were prepared from rat brain as described in detail (Pelkey et al. Neuron 34:127-138 2002). Cellular fractionation of rat brain tissue into nuclear, heavy

mitochondrial, light mitochondrial, microsomal, and cytosolic fractions was performed by differential centrifugation of tissue homogenate in 0.25 M sucrose/10 mM HEPES-NaOH, 1 mM EDTA, pH 7.4 with 2 µg each of aprotinin, pepstatin A, and leupeptin (Sigma, St. Louis, MO) at 4°C. Nuclei were pelleted by centrifugation at 1 000 g for 10 minutes, the supernatant was removed and spun at 3 000 g for 10 minutes to obtain a heavy mitochondrial pellet. The supernatant was removed and spun at 16 000 g for 15 minutes to obtain a light mitochondrial pellet. The supernatant was removed and spun at 100 000 g for 1 hour to obtain a microsomal pellet and the cytosolic fraction. All pellets were then resuspended in RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2.5 mg/ml NaDOC, 1 mM Na_3VO_4 1 mM PMSF, and 2 µg/ml each of protease inhibitors). The light mitochondrial fraction was used in subsequent experiments. For Western blots, 50 µg of total protein was loaded per lane, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-ND2, anti-Cyto1 and anti-ND4 (mouse monoclonals, Molecular Probes Inc., Eugene, OR), anti-PSD95 (mouse monoclonal clone 7E3-1B8, Oncogene Research Products, Cambridge, MA), anti-NR1 (mouse monoclonal clone 54.1, Pharmingen), anti-Src, or anti-synaptophysin (mouse monoclonal, Sigma).

Post-embedding immunogold electron microscopy was carried out. Sprague Dawley rats were anesthetized and perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M phosphate buffer. Parasagittal sections of the hippocampus were cryoprotected in 30% glycerol and frozen in liquid propane. Frozen sections were immersed in 1.5% uranyl acetate in methanol at -90°C, infiltrated with Lowicryl HM-20 resin at -45°C, and polymerized with ultraviolet light. Sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in TBS and 0.1% Triton X-100 (TBST), followed by 10% normal goat serum (NGS) in TBST, primary antibody in 1% NGS in TBST, and immunogold (10 nm; Amersham Pharmacia Biotech) in 1% NGS in TBST plus 0.5% polyethylene glycol. Finally, the sections were stained in uranyl acetate and lead citrate prior to analysis.

In the CNS a prominent subcellular location for Src is in the post-synaptic density (PSD) (Yu et al. Science 275:674-678 1997), a subsynaptic specialization at glutamatergic synapses comprised of α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA-) and NMDA-type glutamate receptors together with scaffolding, signaling and regulatory proteins (Walikonis et al. Journal of Neuroscience 20:4069-4080 2000). Because Src is known to regulate subsynaptic NMDARs (Yu et al. Science 275:674-678

1997), if ND2 is the protein mediating the interaction between NMDARs and the unique domain of Src then ND2 is predicted to be present in the PSD. This was tested by preparing PSD proteins from rat brain homogenates by sequential fractionation and determining whether ND2 was present in this fraction. Characteristic of a bona fide PSD fraction, the fraction which was prepared contained post-synaptic proteins including PSD-95 and NMDA receptor subunit proteins but lacked the pre-synaptic protein synaptophysin (Figure 2A). Figure 2A shows immunoblots of PSD proteins probed with anti-ND2, anti-cytochrome *c* oxidase I (Cyto 1), anti-ND4, anti-PSD95, anti-NR1, anti-Src and anti-synaptophysin as indicated. It was found that ND2 was present in the PSD fraction and the amount of ND2 estimated in this fraction was approximately 15% of that in the total brain homogenate. In contrast to ND2, neither the oxidoreductase protein ND4, another mitochondrially-encoded component of Complex I (J.E. Walker Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov et al. Journal of Molecular Biology 302:455-464 2000; Sazanov et al. Biochemistry 39:7229-7235 2000) nor cytochrome c oxidase subunit 1 (Cyto 1), an inner mitochondrial membrane protein that is part of Complex IV (Marusich et al. Biochim. Biophys. Acta 1362:145-159 1997), was detectable in the PSD fraction. On the other

hand, Cyto 1 and ND4, as well as ND2, were readily detected in proteins from brain mitochondria (Figure 2B). Figure 2B shows immunoblots of mitochondrial proteins prepared by differential centrifugation probed with anti-ND2, anti-Cyto 1 and anti-ND4. Neither NR1 nor NR2A/B was detected in the mitochondrial fraction (data not shown). Although the molecular size of the protein detected by anti-ND2 in the PSD preparation matched that of ND2 in mitochondria, it is conceivable that the protein detected in the PSD preparation was not ND2 but a protein of the same molecular size that was recognized by anti-ND2. However, it was found that incubating anti-ND2 with the antigen to which the antibody was raised prevented the immunoblotting signal (Figure 2C). Figure 2C shows immunoblots of PSD proteins showing the specificity of the N-terminal ND2 antibody by pre-adsorption with the antigenic peptide used to derive the antibody. Morever, it was found that a separate antibody directed towards a distinct epitope in a region of ND2 remote from that of the anti-ND2 epitope also detected ND2, at the correct molecular size, in the PSD preparation, as well as in the mitochondrial preparation (Figure 2D). Figure 2D shows immunoblots of PSD and mitochondrial proteins probed with two independent rabbit polyclonal antibodies directed against two disparate regions of ND2. The N-terminal ND2 antibody was

used for all subsequent experiments illustrated. Thus, ND2 was found in the PSD preparation by two separate antibodies, and this could not be accounted for by a general contamination with mitochondrial proteins because neither Cyto 1 nor ND4 were detected in the PSD.

In addition to examining PSD protein preparations, the presence of ND2 in PSDs was tested for by means of post-embedding immunogold electron microscopy in the CA1 stratum radiatum of rat hippocampus (Petralia et al. Nature Neuroscience 2:31-36 1999; Sans et al. Journal of Neuroscience 20:1260-1271 2000). With this experimental approach the tissue is fixed immediately after the animal is sacrificed and prior to sectioning so that protein localization is preserved. ND2 labeling was found, as visualized by secondary antibody conjugated to 10 nm gold particles, in the PSD and the postsynaptic membrane in dendritic spines of CA1 neurons (Figure 2E), as well as over mitochondria (not illustrated).. Figure 2E shows three representative post-embedding immunogold electron microscopy images of rat hippocampus CA1 synapses, pre-synaptic. Scale bar is 200nm. ND2 labeling was enriched in the post-synaptic membrane approximately 30-fold as compared with the plasma membrane in the remainder of the dendritic spine (0.37 particles per PSD/section versus 0.012, $p<0.05$) and there was

no obvious accumulation of ND2 labeling along the plasma membrane of the dendritic shaft. The ND2 labeling observed in the PSD and post-synaptic membrane could not have been due to labeling in mitochondria because it is known that mitochondria are excluded from dendritic spines (Shepherd et al. Journal of Neuroscience 18(20):8300-8310 1998). Thus, these results indicate that ND2 is present in the biochemically defined PSD protein fraction and is localized at PSDs in CA1 neurons.

EXAMPLE 3

ND2 interacts with Src at the NMDA receptor complex in post-synaptic densities.

Since previous results indicate that ND2 is present in PSDs from brain, it was examined whether ND2 interacts with Src in PSDs. It was found that immunoprecipitating ND2 from the PSD fraction led to co-immunoprecipitation of Src and vice versa (Figure 3A), indicating that ND2 and Src interact post-synaptically at glutamatergic synapses. Figure 3A shows immunoblots of co-immunoprecipitates from PSD preparations probed with anti-ND2 or anti-Src as indicated. Non-specific IgG (either rabbit or mouse) was used as a negative control

for both antibodies. Moreover, Src was pulled from the PSD fraction by the fusion protein ND2.1-GST, but not by either ND2.2- or ND2.3-GST (Figure 3B). Figure 3B shows recombinant ND2.1-GST fusion protein, but not ND2.2-GST, ND2.3-GST, or GST alone, pulls Src form PSD preparations. Thus, as it was found with the Src-ND2 binding in vitro, these results indicate that amino acids 239-321 of ND2 (SEQ ID NO:7) are both necessary and sufficient for ND2 to interact with Src in the PSD.

The hypothesis that ND2 is the protein mediating the interaction between Src and NMDARs requires that, in addition to being present in the PSD and interacting there with Src, ND2 is part of NMDAR complex of proteins. To determine whether ND2 is a component of the NMDAR protein complex, NMDAR complexes were immunoprecipitated from the PSD fraction, using an antibody directed against the core NMDAR subunit NR1 (Dingledine et al. Pharmacology Reviews 51:7-61 1999), and the co-immunoprecipitating proteins were probed with anti-ND2. It was found that ND2 co-immunoprecipitated (Figure 4A), and conversely, immunoprecipitating with anti-ND2 led to co-immunoprecipitation of NR1 (Figure 4A). Figure 4A shows immunoblots of co-immunoprecipitates from PSD preparations probed with anti-ND2 or with anti-NMDA receptor subunit 1 (NR1) as indicated. Non-specific IgG (either rabbit

or mouse) was used as a negative control for both antibodies. Neither ND2 nor NR1 was immunoprecipitated by non-specific IgG, and ND2 did not co-immunoprecipitate with the potassium channel Kv3.1 (Figure 4B), a negative control for non-specific immunoprecipitation of post-synaptic proteins, therefore it was concluded that ND2 is an NMDAR complex protein. Figure 4B shows an immunoblot of co-immunoprecipitates from PSD preparations using anti-GluR2, anti-$GABA_AR\alpha$, anti-$GABA_AR\beta2/3$ and anti-Kv3.1 antibodies to immunoprecipitate. Probe was anti-ND2. Importantly, neither ND4 nor Cyto 1 was detected in co-immunoprecipitates of NR1 (not illustrated) indicating that mitochondrial proteins in general are not components of the NMDAR complex. Moreover, ND2 did not co-immunoprecipitate with GluR2, $GABA_AR\alpha$ or $GABA_AR\beta2/3$ (Figure 4B) indicating that ND2 is not a detectable component of AMPA receptor or γ-aminobutyric acid (GABA) receptor complexes.

Thus, while ND2 is a component of NMDAR complexes it is not generally a component of neurotransmitter receptor complexes in the brain.

EXAMPLE 4

ND2 acts as an adapter protein for Src.

Src40-58 and scrambled Src peptides were biotinylated by incubating with Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) for 30 minutes at room temperature (SEQ ID NO:4, Src protein). The biotinylation reaction was then quenched by the addition of Tris-HCl (pH 8.0) to a final concentration of 20 mM. Purified recombinant fusion proteins (~20 μg each) were dotted onto nitrocellulose and dried overnight. Membranes were blocked with 5% BSA in PBS for 1 hour, after which biotinylated peptides (30 μg/ml) diluted 1:1000 in fresh 5% BSA in PBS were added. The membranes were incubated with the peptides for 1 hour, washed, and probed using a streptavidin-HRP conjugate. Bound probe was then detected on film using an ECL kit.

ND2 acts as an adapter protein for Src. Amino acids 40-58 (SEQ ID NO:4) within the Src unique domain have been implicated in the binding of Src to the interacting protein in the NMDAR complex (Yu et al. Science 275:674-678 1997; Lu et al. Science 279:1363-1368 1998; Yu et al. Nature 396:469-474 1998) and thus, ND2 was predicted to bind to this region

of Src. This prediction was examined in vitro using a peptide with the sequence of amino acids 40-58 (Src40-58;SEQ ID NO:4) which was found to bind directly to ND2.1-GST (Figure 4C) in vitro. In contrast, a peptide with identical amino acid composition, but a scrambled sequence (scrambled Src40-58), did not bind to ND2.1-GST. Neither Src40-58 nor scrambled Src40-58 bound to ND2.2-GST, ND2.3-GST or to GST alone (Figure 4C). Figure 4C shows a dot blot of ND2-GST fusion proteins probed with biotinylated Src40-58 or scrambled Src40-58 peptides followed by streptavidin-HRP conjugate. Furthermore, the effect of Src40-58 on the interaction between Src and ND2 was examined(Figures 4D and 4E). It was found that incubating ND2.1-GST with Src40-58 prevented this fusion protein from pulling down the Src unique domain protein in vitro (Figure 4D). Figure 4D shows a blot of ND2.1-GST probed with boptinylated Src unique domain in the presence of either Src40-58 or scrambled Src40-58 peptides followed by streptavidin-HRP conjugate. On the other hand, scrambled Src40-58 did not affect the interaction between the ND2.1-GST and Src unique domain proteins. Incubating PSD proteins with Src40-58 prevented the co-immunoprecipitation of ND2 by anti-Src but this was not affected by scrambled Src40-58 (Figure 4E). Figure 4E shows immunoblots of co-immunoprecipitates obtained from PSD proteins in the presence

of either Src40-58 or scrambled Src40-58 probed with anti-ND2 or stripped and re-probed with anti-Src. Importantly, Src40-58 did not affect the immunoprecipitation of Src from PSDs. Thus, it was concluded that amino acids 40-58 of Src interact with the region spanned by ND2.1, thereby mediating the binding between the Src unique domain and ND2.

As ND2 alone is not catalytically active (J.E. Walker Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov et al. Journal of Molecular Biology 302:455-464 2000; Sazanove et al. Biochemistry 39:7229-7235 2000), its functional role in the NMDAR complex was investigated. ND2 might be a phosphorylation target for Src, but it was found that ND2 immunoprecipitated from PSD protein fractions was not detectably phosphorylated on tyrosine. Moreover, inclusion of ND2.1-GST did not alter the catalytic activity of Src in vitro (not illustrated) consistent with the binding of ND2 to the unique domain rather than to the regulatory or catalytic domains. Thus, it is unlikely that ND2 is a target of Src or a regulator of Src kinase activity.

However, it was found that the co-immunoprecipitation of Src with NMDARs (Figure 4F, left panel) was suppressed by Src40-58, but not scrambled Src40-58, and by ND2.1 (Figure 4F, right panel) indicating that the association of Src with the NMDAR complexes depends on the interaction with ND2.

Figure 4F, left panel shows immunoblots of co-immunoprecipitates obtained from PSD proteins in the presence of either Src40-58 or scrambled Src40-58. Figure 4F, right panel shows immunoblots of co-immunoprecipitates obtained from PSD proteins in the presence of GST-ND2.1 fusion protein probed with anti-Src or anti-NR1 as indicated. In contrast, the co-immunoprecipitation of ND2 with NMDARs was not affected by Src40-58 (Figure 4G), implying that binding ND2 to Src is not necessary for ND2 to associate with NMDAR complexes. Figure 4G shows immunoblots of co-immunoprecipitates obtained from PSD proteins in the presence of either Src40-58 or scrambled Src40-58 peptides preobed with anti-ND2 or stripped and re-probed with anti-NR1. Taking these results together, it was concluded that ND2 may function as an adapter protein that anchors Src in the NMDAR complex.

EXAMPLE 5

Loss of ND2 in neurons prevents the regulation of NMDA receptor activity by Src.

Fetal rat hippocampal neurons were prepared, cultured, and used for electrophysiological recordings 12-17 days after

plating. Methods for whole cell recordings are described in Pelkey et al. (Neuron 34:127-138 2002).

It was hypothesized that if ND2 is a Src adapter protein then loss of ND2 should prevent the upregulation of NMDAR activity by endogenous Src (Yu et al. Science 275:674-678 1997). This was tested by investigating miniature excitatory post-synaptic currents (mEPSCs) recorded from cultured hippocampal neurons (MacDonald et al. Journal of Physiology (London) 414:17-34 1989). In these neurons the NMDAR-mediated component of mEPSCs is increased by activating endogenous Src with a high-affinity activating phosphopeptide EPQ(pY)EEIPIA (Liu et al. Oncogene 8:1119-1126 1993) and is reduced by applying Src40-58 (Yu et al. Science 275:674-678 1997). It is predicted that each of these effects will be lost by blocking the expression of ND2, if it acts as an adapter protein for Src in the NMDAR complex. In order to suppress ND2 expression, the hippocampal cultures were treated with chloramphenicol to selectively inhibit translation of mitochondrially encoded proteins but not translation of proteins encoded in the nucleus (Ibrahim et al. Journal of Biological Chemistry 251:108-115 1976). After 48 hours treatment with chloramphenicol it was found that the level of ND2 in the cultures was reduced by more than 95%

whereas there was no significant change in the levels of the nuclear encoded proteins examined (Figure 5A). Figure 5A shows immunoblots of total soluble protein obtained from cultured rat hippocampal neurons treated with 50 μg/ml chloramphenicol for 48 hours and probed with anti-ND2, anti-NR1 and anti-Src as indicated. Importantly, chloramphenicol did not affect the level of Src or of the NMDAR subunit NR1 but did suppress the co-immunoprecipitation of Src with the NMDAR complex (Figure 5B), as predicted if ND2 is an adapter protein linking Src to the complex. Figure 5B shows an immunoblot of co-immunoprecipitates obtained from cultured hippocampal neurons, either treated or untreated with 50 μg/ml chloramphenicol for 48 hours and probed with anti-NR1 or anti-Src.

The effect of the 48 hours treatment with chloramphenicol on the ATP levels, mitochondrial membrane potential, viability and general functioning of the hippocampal neurons in culture was examined. It was found that chloramphenicol did not significantly affect the level of ATP levels in the cultures (Figure 5C), consistent with the lack of effect of chloramphenicol treatment for up to 55 hours on ATP levels in other cell types in culture (Ramachandran et al. Proceedings of the National Academy of Science USA 99:6643-6648 2002). Figure 5C shows summary

histograms (left panel) of ATP level or mitochondrial membrane potential ($\Delta\psi M$), as assessed by TMRM fluorescence dequenching (right panel), in cultured hippocampal neurons either untreated or treated 50 μg/ml chloramphenicol for 48 hours. To examine the effect of chloramphenicol on mitochondrial membrane potential ($\Delta\psi M$) in individual neurons, the dequenching of the potentiometric fluorescent cationic dye tetramethylrhodamine methyl ester (TMRM) by the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was monitored (Reers et al. Biochemistry 30:4480-4486 1991). The dequenching response evoked by bath-applied FCCP (2 μM) in neurons from chloramphenicol-treated or control cultures was assessed. It was found that the dequenching response of chloramphenicol-treated neurons was not different from that of untreated neurons (Figure 5C), indicating that $\Delta\psi M$ was not affected by chloramphenicol. Moreover, it was found that neurons treated with chloramphenicol were not distinguishable from untreated neurons in terms of cell number, gross morphology, resting membrane potential, resting intracellular calcium concentration, action potential amplitude, or mEPSC frequency (data not illustrated). Thus, from these data together it was concluded that treatment with chloramphenicol for 48 hours did not detectably compromise the functioning of the

neurons. Nevertheless, it was noted that the intracellular solution used for all whole-cell recordings contained 2 mM Mg-ATP, so that the level of intracellular ATP was equal in all cells throughout the experiments.

In neurons treated with chloramphenicol for 48 hours it was found that the NMDAR component of the mEPSCs was not affected by administering either the EPQ(pY)EEIPIA (SEQ ID NO:5) peptide or the Src40-58 peptide (Figures 5D-F). In contrast, in control experiments administering EPQ(pY)EEIPIA(SEQ ID NO:5) increased the NMDAR component of mEPSCs by 172±28% and application of Src40-58 decreased the NMDAR component to 56±4% (Figures 5D-F). Chloramphenicol was present during the recording periods of the control experiments and therefore the loss of effect of the EPQ(pY)EEIPIA(SEQ ID NO:5) and Src40-58 peptides cannot be attributed to an acute effect of chloramphenicol. Figure 5D shows that the upregulation of NMDAR activity in the presence of the Src activator peptide EPQ(pY)EEIPIA(SEQ ID NO:5), labeled as (pY)EEI (amino acid residues 4-7 of SEQ ID NO:5), is prevented in neurons treated with chloramphenicol for 48 hours. Figure 5E shows that the reduction of NMDA activity in the presence of the Src40-58 peptide is also prevented in neurons treated with chloramphenicol for 48 hours. Composite traces are shown in black, the NMDAR component in dark grey,

and the AMPAR component in light grey. Scale bars are 50ms/10pA. Figure 5F shows a summary histogram of electrophysiology data. NMDA component data were calculated as $Q_{20'}/Q_{2'}$, and AMPA component data were calculated as $A_{20'}/A_{2'}$. A 48 hour chloramphenicol treatment prevents the modulation of NMDAR function by the Src activator peptide (SEQ ID NO:5) and Src40-58 peptides, while neither of these reagents affected the AMPA receptor component of the MEPSCs under the recording conditions used. An * indicates a significant difference, Student's t-test, $p<0.05$. Taking our results together, it is concluded that Src-dependent regulation of the activity of NMDARs depends on expression of ND2 through its anchoring of Src to the NMDAR complex.

EXAMPLE 6

Src40-49 interacts directly with ND2

To detect the binding of ND2.1-GST with Src peptides, the ND2.1-GST fusion protein was purified on glutathione SEPHAROSE. Src40-58, Src40-49, Src49-58, and scrambled Src40-58 peptides (30 mg/ml; synthesized by HSC Peptide Synthesis Facility; all four peptides are schematically depicted in Figure 6A) were biotinylated by incubating with Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) for 30

minutes at room temperature. The biotinylation reaction was then quenched by the addition of Tris-HCl (pH 8.0) to a final concentration of 20 mM. Biotinylated peptides were incubated with ND2.1-GST on beads for 1 hour at 4°C. The beads were washed three times with PBS/0.1% Triton X-100, then resuspended in PBS+SDS-PAGE sample buffer. After brief centrifugation, samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed using a streptavidin-HRP conjugate (Sigma, St. Louis, MO). Bound probe was then detected on film using an ECL kit (Amersham Pharmacia Biotech, Baie d'Urfé, Québec). Figure 6B shows the blot of the ND2.1-GST fusion protein which was probed with biotinylated Src peptides followed by streptavidin-HRP conjugate.

Src40-58, Src40-49, Src49-58, scrambled Src40-58, TAT-Src40-49, and scrambled TAT-Src40-49 peptides were biotinylated by incubating with Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) for 30 minutes at room temperature. The biotinylation reaction was then quenched by the addition of Tris-HCl (pH 8.0) to a final concentration of 20 mM. Purified recombinant fusion proteins (~20 µg each) were dotted onto nitrocellulose and dried overnight. Membranes were blocked with 5% BSA in PBS (pH 7.5) for 1 hour, after which biotinylated peptides (30 µg/ml) diluted

1:1000 in fresh 5% BSA in PBS were added. The membranes were incubated with the peptides for 1 hour, washed, and probed with streptavidin-HRP conjugate. Bound probe was then detected on film using an ECL kit. Figure 6C shows the dot blots of ND2.1-GST fusion proteins probed with biotinylated Src peptides followed by streptavidin-HRP conjugate.

EXAMPLE 7

TAT-Src40-49 (TSUDAPI-1) reduces pain behavior

Male Sprague-Dawley rats 150-200 g were used for all experiments. Rats were housed in pairs, maintained on a 12/12 hour light/dark cycle, and allowed free access to food and water. All experiments were conducted during 10 am and 5pm.

Peptide Src40-49Tat (TSUDAPI-1; SEQ ID NO:2) or Tat alone (amino acid residues 1-11 of SEQ ID NO:2) was dissolved in sterilized saline. Peptide or saline was injected intravenously at a volume 1ml/Kg into rat's tail 45 minutes before behavioral testing. Injections were done under brief halothane anesthesia and rats were returned to the cages after injections.

The formalin test was performed as previously described (Liu et al. European Journal of Pharmacology 408(2):143-152 2000). Rats were placed in a plexiglass observation chamber

for an initial 20 minutes to allow acclimatization to the testing environment. Formalin 2.5% was injected subcutaneously in a volume of 50 ml into the plantar aspect of the hind paw. Following injections, rats were returned to the observation chamber and monitored for flinching behaviors (lifting, shaking and overt flinching with a ripple over the haunch) and biting/licking time. Two rats in adjacent chambers were observed at one time, with observations occurring in alternate 2 minute bins. Recorded episodes were not corrected, thus values represent about half of the total behaviors expressed.

Figures 7A-D show the effect of Src40-49Tat (0.1pmol) on 2.5% formalin induced flinching or biting/licking behaviors. Peptides or saline controls were injected 45 minutes before behavioral testing. Figure 7B shows measurement of flinching behaviors observed within an hour. Figure 7A shows the cumulative flinches in different phases observed within the hour. P1 represents a time period of 0-8 minutes; P2A represents a time period of 12-28 minutes and P2B represents a time period of 32-60 minutes. Values depict means (n=7, Src40-49Tat; n=20, saline). $P<0.05$, $P<0.01$ with student t test compared to saline control. Figure 7D shows measurement of the time of each biting/licking behavior observed within an hour. Figure 7C shows the cumulative biting/licking

behaviors in different phases observed within the hour.P1 represents a time period of 0-8 minutes; P2A represents a time period of 12-28 minutes and P2B represents a time period of 32-60 minutes. Values depict means (n=7, Src40-49Tat; n=20, saline). $P<0.05$, $P<0.01$ with student t test compared to saline control.

Figures 8A-D show the effect of HIV-Tat (1pmol/g) on 2.5% formalin induced flinching or biting/licking behaviors. Peptides or saline controls were injected 45 minutes before behavioral testing. Figure 8B shows measurement of flinching behaviors observed within an hour. Figure 8A shows the cumulative flinches in different phases observed within the hour. P1 represents a time period of 0-8 minutes; P2A represents a time period of 12-28 minutes and P2B represents a time period of 32-60 minutes. Values depict means (n=7, HIV-Tat; n=20, saline). $P<0.05$, $P<0.01$ with student t test compared to saline control. Figure 8D shows measurement of the time of each biting/licking behavior observed within an hour. Figure 8C shows the cumulative biting/licking behaviors in different phases observed within the hour.P1 represents a time period of 0-8 minutes; P2A represents a time period of 12-28 minutes and P2B represents a time period of 32-60 minutes. Values depict means (n=7, HIV-Tat; n=20, saline). $P<0.05$, $P<0.01$ with student t test compared to

saline control. As compared to HIV-Tat alone and the saline control, the Src40-49Tat peptide is shown to reduce pain behaviors over a time period of an hour.

EXAMPLE 8

ND2-Src interaction in multiple tissues

Total soluble protein was prepared from pre-weighed rat tissues by homogenization at 4°C in 0.25 M sucrose/10mM HEPES-NaOH, 1mM EDTA, pH 7.4 with 2μg/ml each of aprotinin, pepstatin A, and leupeptin. Following brief configuration of the samples at 4 000g, NP-40 was added to 1% (vol/vol) to the cleared supernatants. After incubation for 10 minutes, the protein concentration of the samples was determined by detergent compatible protein assay (BioRad Laboratories, Mississauga, Ontario) and equilibrated. The solubilized proteins were centrifuged briefly at 14 000g to remove insoluble material and then incubated with 5μg of either anti-ND2 (rabbit polyclonal from Dr. R.F. Doolittle, UCSD, CA; described in Mariottini et al. PNAS USA 83:1563-1567 1986), anti-Src (mouse monoclonal clone 327 from J. Bolen, DNAX, Palo Alto, CA) or control, non-specific rabbit or mouse IgG (Sigma) overnight at 4°C. Immune complexes were isolated by the addition of 10μl of protein G-SEPHAROSE beads followed by incubation for 2 hours at 4°C. Immunoprecipitates were then washed three times with RIPA buffer, re-suspended in

RIPA buffer + SDS-PAGE sample buffer and boiled for 5 minutes. The samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-ND2, anti-Src or anti-Fyn (mouse monoclonal clone 25, Pharmingen, Mississauga, Ontario). Bound antibody was then detected on film using appropriate secondary antibody/HRP conjugates and an ECL kit (Amersham Pharmacia Biotech). For control immunoprecipitations under denaturing conditions, SDS was added to the initial protein samples to a final concentration of 0.4% and the samples were boiled for 5 minutes and rapidly cooled to 4°C prior to the addition of the antibodies used for immunoprecipitation. In addition, pre-adsorption of the anti-ND2 antibody with antigenic peptide prevented antibody signal detection on immunoblots.

Non-receptor tyrosine kinase Src and ND2 are both expressed in cells of multiple, diverse tissues. Illustrative, albeit non-limiting, examples are peripheral nervous system tissue, central nervous system tissue, heart, intestine, kidney, liver, lung, pancreas, skeletal muscle, spleen, testis, bone, skin and brain. The data presented in Figures 10A-B shows that ND2 and Src interact in multiple, diverse tissues. Immunoblots of co-immunoprecipitates from various tissues (Figure 10A) and tissue homogenates (Figure 10B) probed with anti-ND2, anti-Src, or anti-Fyn as

indicated. Tissues: B-brain; H-heart; I-intestine; K-kidney; Liv-liver; Lu-lung; P-pancreas; Sk-skeletal muscle; Sp-spleen and T-testis. Non-specific IgG applied to liver homogenate was used as a negative control for co-immunoprecipitation. Immunoblotting of Fyn protein from brain was used as a positive control for the anti-Fyn antibody. In these experiments the cell lysates were prepared using non-denaturing conditions, but when denaturing conditions were used to prepare the proteins, no co-immunoprecipitation of Src by anti-ND2 or of anti-Src was found (data not illustrated).

IN SUMMARY

The main criteria for identifying ND2 as the protein mediating the interaction between NMDARs and the unique domain of Src, as inferred from previous work (Ali et al. Current Opinion in Neurobiology 11:336-342 2001; Yu et al. Science 275:674-678 1997) are as follows: ND2 must bind directly to the unique domain of Src through amino acids 40-58(specifically 40-49;SEQ ID NO:1); this binding must be prevented by the Src40-58 (specifically 40-49) peptide; ND2 must be present at excitatory synapses and must be a component of the NMDAR complex; and lack of ND2 must prevent the upregulation of NMDAR activity by endogenous Src.

ND2 was first considered as a potential Src unique domain-binding protein when overlapping clones of ND2 in two separate yeast two-hybrid experiments were isolated. Subsequently, the direct interaction of the Src unique domain and ND2 was confirmed through in vitro binding assays using recombinant proteins. Through these experiments the ND2.1 region was identified as necessary and sufficient for interacting with the Src unique domain. ND2.1 bound directly to the Src40-58 (specifically 40-49)peptide and the in vitro binding of the Src unique domain to ND2.1 was prevented by Src40-58 (specifically 40-49). Src and ND2 co-immunoprecipitated with each other in brain homogenates and PSD protein preparations. The co-immunoprecipitation was prevented by Src40-58(specifically 40-49), implying that the Src-ND2 interaction identified in vitro may occur in vivo. In addition to finding ND2 in PSD protein preparations, ND2-immunoreactivity was found by immunogold electron microscopy in PSDs in the CA1 hippocampus. Moreover, co-immunoprecipitation experiments indicated that ND2 is a component of the NMDAR complex and that the Src-ND2 interaction is required for the association of Src, but not ND2, with NMDARs. It was found that depleting ND2 suppresses Src association with the NMDAR complex and prevents the upregulation of NMDAR function by activating endogenous Src

at excitatory synapses. Src40-49 (SUDAPI-1;SEQ ID NO:1)was identified as the specific peptide that interacts with ND2 as Src50-58 alone did not interact with ND2. Finally, it was found that TAT-Src40-49 (TSUDAPI-1;SEQ ID NO:2) as administered to rats reduced pain behavior in the formalin test. These multiple, converging lines of evidence lead to the conclusion that ND2 is the protein mediating the interaction between NMDARs and the unique domain of Src.

ND2 is mitochondrially encoded and translated, and yet it is found within the PSDs of glutamatergic synapses in the brain. The other mitochondrial proteins examined, ND4 and Cyto 1, were not detected in the PSD fraction implying that this fraction is not contaminated non-specifically by mitochondrial proteins. Further, ND2-immunoreactivity by immunogold electron microscopy was found within structurally-identified PSDs in dendritic spines of CA1 neurons. In this preparation, proteins are immobilized by tissue fixation precluding the possibility that ND2 could have relocated from the mitochondria to the PSD during processing. Moreover, because dendritic spines are devoid of mitochondria (Shepherd et al. Journal of Neuroscience 18(20):8300-8310 1998) the ND2 immunoreactivity cannot be accounted for by mitochondria abutting the PSD. Taken together these findings indicate that ND2, but not the entire Complex I, is normally present

within the PSD. The PSD contains many enzymes that may be involved in regulating synaptic functioning (P. Siekevitz Proceedings of the National Academy of Science USA 82:3494-3498 1985) including glycolytic enzymes capable of generating ATP (Wu et al. Proceedings of the National Academy of Science USA 94:13273-13278 1997). However, without other components of Complex I it is unlikely that ND2 functions catalytically within the PSD.

Thus, in addition to its localization in mitochondria and function as a component of Complex I, the present results indicate that ND2 has a second location and function in outside the mitochondria. Mitochondria are intimately linked to overall cellular functioning through generation of ATP by oxidative phosphorylation. Mitochondria are also known to be key for sequestration of intracellular calcium (D.D. Friel Cell Calcium 28:307-316 2000; R. Rizzuto Current Opinion in Neurobiology 11:306-311) and to participate in programmed cell death (Gorman et al. Developmental Neuroscience 22:348-358 2000; M.P. Mattson National Review of Molecular and Cellualr Biology 1:120-129 2000). Some mitochondrial proteins are known to be present at extra-mitochondrial sites (Soltys et al. Trends in Biochemical Science 24:174-177 1999; Soltys et al. International Review of Cytology 194:133-196 1999). But, the experiments described herein indicate a new type of

function for a mitochondrial protein outside this organelle, that is ND2 acts as an adapter protein that anchors Src within the NMDAR complex, where it thereby allows Src to upregulate NMDAR activity.

Upregulating the activity of NMDARs is a major function of Src in neurons in the adult CNS (Lu et al. Science 279:1363-1368 1998; Pelkey et al. Neuron 34:127-138 2002; Huang et al. Neuron 29:485-496 2001) and this mediates the induction of long-term potentiation (LTP) of excitatory synaptic transmission in CA1 neurons in the hippocampus (Ali et al. Current Opinion in Neurobiology 11:336-342 2001). The findings described herein imply that the ND2-Src interaction is essential for LTP induction as LTP in CA1 neurons is prevented by Src40-58 and by anti-Src1, an antibody that recognizes this amino acid sequence within the Src unique domain and which prevents the Src unique domain interaction with ND2.1 in vitro (J.R.G., M.W.S. unpublished observations). LTP at Schaffer collateral-CA1 synapses is the prototypic example of NMDAR-dependent enhancement of excitatory synaptic transmission, which is observed at numerous types of glutamatergic synapses throughout the CNS (Malenka et al. Science 285:1870-1874 1999). In addition, Src has been implicated in NMDAR-dependent seizures (Sanna et al. Proceedings of the National Academy of Science 97:8653-

8657 2000), chronic pain (Guo et al. Journal of Neuroscience 22:6208-6217 2002) and neurotoxicity (Pei et al. Journal of Cerebral Blood Flow Metabolism 21:955-963 2001). Thus, the discovery of the Src-ND2 interaction at NMDARs, which is disclosed herein, defines a protein-protein interaction of general relevance to regulation of neuronal function, synaptic plasticity, and pathophysiology in the CNS.

Additionally, by showing an extramitochondrial action for a protein encoded in the mitochondrial genome a previously unsuspected means by which mitochondria regulate cellular function has been identified. Because ND2 and Src are broadly expressed, the interaction of ND2 with the Src unique domain may be of general relevance for control of Src signaling (Example 8 and Figures 10A-B).

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.
It is to be understood that while a certain form of the invention is illustrated, it is not to be limited to the specific form or arrangement herein described and shown. It will be apparent to those skilled in the art that various

changes may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown and described in the specification. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned, as well as those inherent therein. The oligonucleotides, peptides, polypeptides, biologically related compounds, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.